

INHIBITION OF BACTERIA ISOLATED FROM NINH THUAN GRAPES BY ORGANIC ACIDS

Nguyen Thi Hanh¹, Nguyen Thi Huong¹, Ho Phu Ha^{1,*}, Nguyen Xuan Phuong¹,
Pham Anh Tuan²

¹ Hanoi University of Science and Technology, 1 Dai Co Viet Road, Hanoi, Vietnam

² Vietnam Institute for Agricultural Engineering and Post Harvest Technology,
126 Trung Kinh Road, Hanoi, Vietnam

*Email: ha.hophu@hust.edu.vn

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ABSTRACT

Ninh Thuan grapes are famous specialty of Vietnam. After harvesting, they are very susceptible to damage if not preserved in time. This study aimed to find the minimal inhibitory concentration and minimal bactericidal concentrations of organic acids, such as lactic acid, citric acid, and ascorbic acid towards seven bacteria previously isolated from Ninh thuan grapes (*Empedobacter brevis*, *Citrobacter* sp., *Enterobacterium ludwigii*, *Bacillus cereus*, *Flavobacterium* sp., *Pseudomonas oryzihabitans* and *Bacillus thuringiensis*) in order to apply in pretreatment of grapes prior to storage. The Minimal Inhibitory Concentration (MIC) was determined by the binary dilution method. 0.1 ml of each dilution of test acids with initial concentration of 3 % was mixed with 0.1 ml of bacteria (c.a. 5.10^5 CFU/ml), in 96-culturing-well plate. Inoculated plate was incubated for 24 hours at temperature of 37 °C. Optical density was measured at 620 nm wavelength by a Microplate reader device. For Minimal Bactericidal Concentration (MBC) determination, 0.1 ml of bacteria (c.a. 5.10^5 CFU/ml) was mixed with 0.4 ml liquid medium of Tryptone Glucose Agar (TGA) and of 0.5 ml acid at above concentrations, culturing within 24 hours at temperature of 37 °C. After 24 hours, they were dropped onto TGA agar plates, and cultured for 24 hours at 37 °C. The results showed that, the MIC and MBC of citric acid toward *Empedobacter brevis* were 0.12 mg/ml and 0.12 mg/ml; *Citrobacter* sp. were 0.9 mg/ml and 3.8 mg/ml; *Enterobacterium ludwigii* were 0.45 mg/ml and 1.9 mg/ml; *Bacillus cereus* were 0.9 mg/ml and 0.9 mg/ml; *Flavobacterium* sp. were 0.12 mg/ml and 0.45 mg/ml; *Pseudomonas oryzihabitans* were 0.12 mg/ml and 0.45 mg/ml and *Bacillus thuringiensis* were 0.12 mg/ml and 0.9 mg/ml, respectively. Similarly, MIC and MBC of lactic acid toward: *Empedobacter brevis* were 0.12 mg/ml and 0.23 mg/ml; *Citrobacter* sp. were 0.9 mg/ml and 1.9 mg/ml; *Enterobacterium ludwigii* were 0.45 mg/ml and 0.9 mg/ml; *Bacillus cereus* were 0.23 mg/ml and 0.45 mg/ml; *Flavobacterium* sp. were 0.12 mg/ml and 1.9 mg/ml; *Pseudomonas oryzihabitans* were 0.23 mg/ml and 1.9 mg/ml and *Bacillus thuringiensis* were 0.12 mg/ml and 1.9 mg/ml, respectively. Ascorbic acid was found to be inefficient for use as antimicrobial agent against isolated bacteria. The above results suggested that citric and lactic acids could be used at maximal concentrations of 3.8 mg/ml and 1.9 mg/ml, respectively to suppress bacteria from

grapes. This finding would contribute to develop method for pretreatment of grapes in fresh grape preservation techniques.

Keywords: minimal inhibitory concentration, minimal bactericidal concentration, Ninh Thuan grapes, organic acids.

1. INTRODUCTION

Grapes have been grown in Vietnam for a long time, but only centralized in certain areas to certain favorable climate conditions. In addition, there are problems of post-harvest losses due to transportation and decay-causing activity of microorganisms. Therefore, there is a need to study of methods to prolong post harvest storage. There is a number of grape preservation including: refrigeration preservation, controlled atmosphere (CA), modified atmosphere packaging (MAP), SO₂ dip, chitosan douse, irradiation UV etc. [1]. Different methods are normally used in combination to increase their efficiency. Step of washing and pre-treatment with antimicrobial agents, including organic acids, is applied to support grapes preservation methods.

Grapes steeped in acetic acid at 0.27 % for 30 minutes, stored at 2 – 5 °C to reduce some types of mold such as *Penicilium* spp. and *Botrytis cinerea* can be stored for 6 weeks without affecting the quality of the grapes. Using acetic acid at a concentration of 8 mg/L combined with MAP method extends storage time up to 74 days at 0 °C, reducing the decaying rate from 94 % to 2 % [2]. Venditti et al. found that, grapes steeped in 75 µl/L acetic acid solution for 15 minutes and stored at 0 – 5 °C in 95 % humidity extended storage time of up to 8 weeks without compromising the quality of the grapes [3].

There is a limited number of literatures in Vietnam reporting method of grape preservation. Contaminated bacteria have been previously isolated from Ninh thuan grapes (unpublished data). This study is aimed to find the minimal inhibitory concentration and minimal bactericidal concentrations of organic acids, such as lactic acid, citric acid, and ascorbic acid toward isolated bacteria. Obtained results could be potential for applying in pretreatment of grapes for further storage.

2. MATERIALS AND METHODS

2.1. Materials

Seven bacteria were isolated and identified in previous studies: *Empedobacter brevis*, *Citrobacter* sp., *Enterobacterium ludwiggi*, *Bacillus cereus*, *Flavobacterium* sp., *Pseudomonas oryzihabitans*, *Bacillus thuringiensis* (unpublish data) Lactic acid, citric acid, ascorbic acid of food grade were used in this study.

2.2. Methods

2.2.1. Method of determining the antibacterial activity of organic acids.

Antibacterial activity of organic acids was determined by well diffusion method [4]. Three kinds of organic acids, that are lactic acid, citric acid and ascorbic acid, were diluted with pasteurized distilling water to the concentrations of 1 %. Bacteria were cultured in Tryptone Glucose Agar (TGA) liquid media, at 37 °C for 24 hours. 0.1 ml of the culture was spread

evenly on the TGA agar surface until it was dry. Four holes with diameter of 6mm were perforated. 0.1 ml of each above acid solution was placed into the holes. Sterile distilled water and clinecid antibiotic of 1 % concentration were used as control. The acids were allowed to diffuse in to agar medium in the refrigerator at 4 °C for 3 hours. Then the plates were transferred to the cabinet at 37 °C, cultured for 24 h. Diameters of antibacterial zones were recorded.

2.2.2. Method of determining the Minimal Inhibitory Concentration (MIC)

A range of binary acid dilutions with sterile distilled water were obtained from original concentration of 3 %: One day culture of test bacteria was diluted with saline water to the final count of 5×10^5 CFU/ml. 0.1 ml serial diluted organic acid solutions and 0.1 ml of bacteria culture were placed in 96 well plate, cultured at 37 °C for 24 h. Optical density was measured in Microplate Reader using filter at 620 nm [5]. A positive control containing the bacterial culture without the organic acid and a negative control containing the Muller Hilton Broth (MHB) and organic acid were performed in the same conditions. The MIC was determined as the lowest concentration showing no growth.

2.2.3. Method of determining the Minimal Bactericidal Concentration (MBC)

MBC was determined by transferring 10 µl solution from the 96-well plate containing the bacteria after culturing at 37 °C for 24 h in Section 2.2.2. The solutions were separately dropped on TGA plate. A positive control containing the bacterial culture without the organic acid and a negative control containing the MHB and organic acid were performed in the same conditions. The plates were incubated at 37 °C for 24 hours. The MBC was identified as the lowest concentration showing no bacterial growth on agar plates [6].

3. RESULTS AND DISCUSSION

3.1. Antibacterial activities of organic acids against bacteria isolated from Ninh Thuận grapes

Firstly, antibacterial activity of three organic acids - lactic acid, citric acid, and ascorbic acid - was studied. Example of inhibition zones to *Bacillus thuringiensis* are presented in Fig. 3.1. Diameters of inhibition zones of organic acids towards isolated bacteria are presented in Table 3.1. As shown in Fig. 3.1, there was no clear zone around the well with water, however, there were inhibition areas around wells with acids which meant that the afore mentioned acids were all able to inhibit the isolated bacteria from grape at the concentration of 1 %.

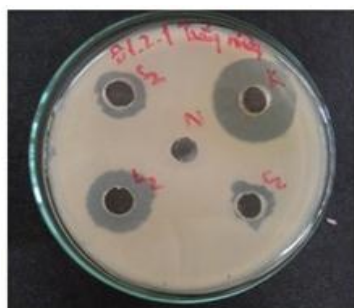


Fig.3.1: The inhibition zone of organic acid at concentration of 1% against *Bacillus thuringiensis*

Note: S –ascorbic acid; L –lactic acid; C –citric acid;

N – water; K – clinecid 30 µg

Table 3.1. Antimicrobial activity of organic acids at 1% toward isolated bacteria.

| Bacterial types | Diameter of Antimicrobial zone (mm) | | |
|----------------------------------|-------------------------------------|-------------|---------------|
| | Lactic acid | Citric acid | Ascorbic acid |
| <i>Empedobacter brevis</i> | 13,0 ± 2,0 | 14,0 ± 2,0 | 8,0 ± 3,0 |
| <i>Citrobacter sp.</i> | 10,0 ± 1,0 | 9,0 ± 3,0 | 2,0 ± 2,0 |
| <i>Enterobacterium ludwigii</i> | 8,0 ± 2,0 | 7,0 ± 1,0 | 6,0 ± 3,0 |
| <i>Bacillus cereus</i> | 10,0 ± 1,0 | 14,0 ± 1,0 | 5,0 ± 2,0 |
| <i>Flavobacterium sp.</i> | 11,0 ± 1,0 | 12,0 ± 2,0 | 8,0 ± 3,0 |
| <i>Pseudomonas oryzihabitans</i> | 13,0 ± 2,0 | 11,0 ± 4,0 | 5,0 ± 1,0 |
| <i>Bacillus thuringiensis</i> | 9,0 ± 1,0 | 15,0 ± 1,0 | 6,0 ± 2,0 |

Organic acids have been used for inhibition of spoilage and pathogenic organisms. According to Manab et al. [7], of whey protein based edible film containing organic acids demonstrated antimicrobial activity against *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Escherichia coli* and *Salmonella sp.* Whey protein based edible film containing benzoic acid had the biggest inhibition zone against those four microorganisms tested (that is, 11.76 mm), while the one containing lactic acid showed the smallest inhibition zone (that is, 10.44 mm). The inhibition zone of the film containing propionic acid was 10.94 mm and was smaller than the one containing benzoic acid; however this inhibition zone was still bigger than the one shown by the film containing acetic acid (10.45 mm). Although activity of organic acids in our study was shown on spoilage bacteria isolated from grapes, our results were comparable with this study. In our investigation, in general, *Empedobacter brevis* was the most sensitive to acids among bacteria isolated from Ninh Thuan grapes. Whereas *Citrobacter sp.* was the most resistant to test acids. Lactic acid demonstrated the highest antagonistic against *Empedobacter brevis* and *Pseudomonas oryzihabitans*, whereas citric acid could inhibit *Bacillus thuringiensis* the most. Antimicrobial capability of ascorbic acid was the lowest among test organic acids, therefore, lactic acid and citric acid were used for further experiments.

3.2. The Minimal Inhibitory Concentration (MIC) of latic acid and citric acid to bacteria isolated from Ninh Thuan grapes

The Minimal Inhibitory Concentration (MIC) of latic acid and citric acid to bacteria isolated from Ninh Thuan grapes were determined and presented in Table 3.2.

Table 3.2. MIC of latic acid and citric acid to bacteria isolated from Ninh Thuan grapes.

| Bacterial types | MIC value (mg/ml) | |
|----------------------------------|-------------------|-------------|
| | Lactic acid | Citric acid |
| <i>Empedobacter brevis</i> | 0.12 | 0.12 |
| <i>Citrobacter sp.</i> | 0.9 | 0.45 |
| <i>Enterobacterium ludwigii</i> | 0.45 | 0.45 |
| <i>Bacillus cereus</i> | 0.45 | 0.9 |
| <i>Flavobacterium sp.</i> | 0.12 | 0.12 |
| <i>Pseudomonas oryzihabitans</i> | 0.23 | 0.12 |
| <i>Bacillus thuringienis</i> | 0.12 | 0.12 |

Table 3.2 shows the results of the Minimum Inhibitory Concentration of the two acids to seven types of bacteria. The highest MIC value of citric acid for *B.cereus* was 0.9 mg/ml and the

highest MIC values of lactic acid for bacteria *Citrobacter* sp. was 0.9 mg/ml. The lowest MIC value of citric acid and lactic acid for three bacteria: *Empedobacter brevis*, *Flavobacterium* sp., *Bacillus thuringiensis* was 0.12 mg/ml.

Indeed, Sheung woo Seo et al. [8] reported that organic acids can inhibit the growth of pathogenic bacteria and spoilage organisms. *Escherichia coli* O157:H7 or *Salmonella Typhimurium* was treated with a 1 % citric acid, 2 % citric acid. The addition of 1 or 2 % citric acid caused 0.28–0.57 log reductions of *E. coli* O157: H7 or *S. Typhimurium* within 60 min. In conclusion, citric acid act synergistically showed its potential of an effective hurdle for the inactivation of foodborne pathogens. In addition, Ye-Won In et al. [9] determined the antimicrobial activities of acetic acid, citric acid and lactic acid against four *Shigella* species: *S. sonnei*, *S. flexneri*, *S. boydii* and *S. dysenteriae*. MICs of acetic acid and citric acid against *Shigella* were 200 and 300 ppm, respectively. But *S. sonnei* was 400 ppm. Lactic acid (0.5 %) in tryptic soya broth inhibited the growth of all *Shigella* species. Citric acid weakly inhibited the growth of *S. flexneri*, but it strongly inhibited the growth of *S. dysenteriae*, resulting in a 5-log reduction.

3.3. The Minimal Bactericidal Concentration (MBC) of latic acid and citric acid to bacteria isolated from Ninh Thuan grapes

The Minimal Bactericidal Concentration (MBC) acids to bacteria are shown in Table 3.3, image of MBC test was presented as an example in Figure 3.2. The Table 3.3 showed that *Citrobacter* sp. had the ability to withstand in acid environment higher than other bacteria (concentrations to lactic acid and citric acid were 1.9 mg/ml and 3.8 mg/ml respectively), and *Empedobacter brevis* was less resistant bacteria (MBC of lactic acid and citric acid were 0.23 mg/ml and 0.12 mg/ml respectively).

Table 3.3. Minimal Bactericidal Concentration (MBC) of latic acid and citric acid to bacteria isolated from Ninh Thuan grapes.

| Bacteria types | MBC value (mg/ml) | |
|----------------------------------|-------------------|-------------|
| | Lactic acid | Citric acid |
| <i>Empedobacter brevis</i> | 0.23 | 0.12 |
| <i>Citrobacter</i> sp. | 1.9 | 3.8 |
| <i>Enterobacterium ludwigii</i> | 1.9 | 1.9 |
| <i>Bacillus cereus</i> | 0.45 | 0.9 |
| <i>Flavobacterium</i> sp. | 1.9 | 0.45 |
| <i>Pseudomonas oryzihabitans</i> | 1.9 | 0.45 |
| <i>Bacillus thuringiensis</i> | 1.9 | 0.9 |

Indeed, Yilmaz [10] reported that The MICs and MBCs of boric acid were obtained as 3.80 mg/mL, 3.80 mg/mL, 7.60 mg/mL, and 7.60 mg/mL against the bacterial activities of *Staphylococcus aureus*, *Acinetobacter septicus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, respectively.

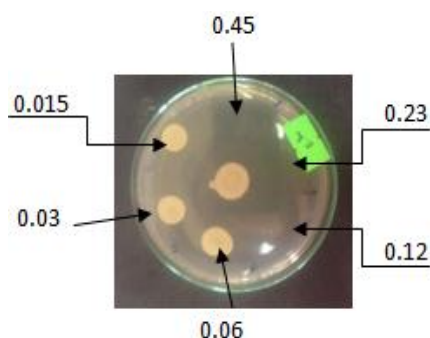


Figure 3.2: Minimal Bactericidal Concentration (MBC)
of citric acid to *Empedobacter brevis* (mg/ml)

Figure 3.2 shows the growth of *Empedobacter brevis* at citric acid concentrations of 0.015; 0.03; 0.06; 0.12; 0.23 and 0.45 mg/ml anticlockwise and control sample (sterile distilled water) in the center. At citric acid concentrations of 0.12; 0.23 and 0.45 mg/ml, we did not observed antimicrobial zone. As a result, MBC of citric acid to *Empedobacter brevis* was determined as 0.12 mg/ml. For total inhibition effect on bacteria from Ninh Thuan grapes, it is recommended to use lactic acid at concentrations of 1.9 mg/ml or citric acid at concentration of 3.8 mg/ml.

In numerous studies, organic acids were used to control fruit decay refers, within postharvest pathology, to the use of disinfecting agents that are allowed in food industries and are applied on the fruit and vegetable surface because of their antimicrobial properties [11, 12]. Acetic, citric, malic, tartaric and propionic acids are classified by the FDA as GRAS compounds. They can be added to food matrices as preservatives, since they can act as antimicrobials, preventing food spoilage from bacteria and fungi, or as antioxidants, slowing or preventing changes in color, flavor, or texture and delaying rancidity.

4. CONCLUSION

The study has found the Minimal Inhibitory Concentration and Minimal Bactericidal concentration of citric acid and lactic acid against microorganisms isolated from Ninh Thuan grapes which ranged from 0.12 mg/ml to 3.8 mg/ml and 0.12 mg/ml to 1.9 mg/ml, respectively. It suggested that citric acid and lactic acid could be used at concentrations of 3.8 mg/ml and 1.9 mg/ml, for pretreatment of Ninh Thuan grapes in post harvest storage.

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TÓM TẮT

KHẢ NĂNG KHÁNG VI KHUẨN PHÂN LẬP TỪ NHO NINH THUẬN CỦA MỘT SỐ AXIT HỮU CƠ

Nguyễn Thị Hạnh¹, Nguyễn Thị Hương¹, Hồ Phú Hà^{1,*}, Nguyễn Xuân Phương¹,
Phạm Anh Tuấn²

¹Trường Đại học Bách khoa Hà Nội, Số 1, Đại Cồ Việt, Hà Nội

²Viện Cơ điện nông nghiệp và Công nghệ sau thu hoạch, Số 126 Trung Kính, Hà Nội

*Email: ha.hophu@hust.edu.vn

Nho Ninh Thuận là một đặc sản nổi tiếng của nước ta. Sau khi thu hoạch chúng rất dễ bị hư hỏng nếu không được bảo quản kịp thời. Nghiên cứu này tiếp tục tiến hành tìm nồng độ ức chế tối thiểu và nồng độ diệt khuẩn tối thiểu của axit lactic, axit citric, axit ascorbic đối với 7 loại vi khuẩn đã được phân lập từ nho Ninh Thuận trước đây (*Empedobacter brevis*, *Citrobacter* sp.,

Enterobacterium ludwigii, *Bacillus cereus*, *Flavobacterium* sp., *Pseudomonas oryzihabitans* và *Bacillus thuringiensis*) để ứng dụng trong tiền xử lí trước khi bảo quản nho Ninh Thuận. Nồng độ ức chế tối thiểu (MIC) được xác định bằng phương pháp pha loãng nhị phân. Với nồng độ axit ban đầu là 3 % và dịch vi khuẩn (5×10^5 CFU/ml). Mật độ tế bào còn lại được đo quang ở bước sóng 620 nm. Đối với nồng độ diệt khuẩn tối thiểu (MBC), lấy dịch vi khuẩn sau khi nuôi ở thí nghiệm xác định MIC nhỏ giọt lên các đĩa môi trường thạch TGA nuôi trong vòng 24 giờ ở 37 °C. Kết quả cho thấy MIC và MBC tương ứng của axit citric đối với *Empedobacter brevis* là 0,12 mg/ml và 0,12 mg/ml; *Citrobacter* sp. là 0,9 mg/ml và 3,8 mg/ml; *Enterobacterium ludwigii* là 0,45 mg/ml và 1,9 mg/ml; *Bacillus cereus* là 0,9 mg/ml và 0,9 mg/ml; *Flavobacterium* sp. là 0,12 mg/ml và 0,45 mg/ml; *Pseudomonas oryzihabitans* là 0,12 mg/ml và 0,45 mg/ml và *Bacillus thuringiensis* là 0,12 mg/ml và 0,9 mg/ml. Tương tự, MIC and MBC của axit lactic là: *Empedobacter brevis* là 0,12 mg/ml và 0,23 mg/ml; *Citrobacter* sp. là 0,9 mg/ml và 1,9 mg/ml; *Enterobacterium ludwigii* là 0,45 mg/ml và 0,9 mg/ml; *Bacillus cereus* là 0,23 mg/ml và 0,45 mg/ml; *Flavobacterium* sp. là 0,12 mg/ml và 1,9 mg/ml; *Pseudomonas oryzihabitans* là 0,23 mg/ml và 1,9 mg/ml và *Bacillus thuringiensis* là 0,12 mg/ml và 1,9 mg/ml. Axit ascorbic cho thấy không hiệu quả để làm tác nhân kháng vi khuẩn đã phân lập. Kết quả trên cho thấy axit citric và axit lactic có thể sử dụng nồng độ tương ứng là 3,8 mg/ml và 1,9 mg/ml để tiêu diệt được vi khuẩn phân lập từ nho. Nghiên cứu này đã góp phần phát triển phương pháp tiền xử lí nho Ninh Thuận trong kĩ thuật bảo quản nho sau thu hoạch.

Từ khóa: nồng độ ức chế tối thiểu, nồng độ diệt khuẩn tối thiểu, nho Ninh Thuận, axit hữu cơ.